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PRINCIPAL INVESTIGATOR: Fang Liu, Ph.D.

CONTRACTING ORGANIZATION: Rutgers University
New Brunswick, New Jersey 08901-8559

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Fang Liu, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Rutgers University
New Brunswick, New Jersey 08901-8559

E-Mail: fangliu@cabm.rutgers.edu

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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*
Transforming growth factor-ß (TGF-ß) potently inhibits cell proliferation by causing cell cycle arrest at the G1 phase. Smad proteins mediate the TGF-ß antiproliferative responses by regulating the expression of several cell cycle components. We have recently discovered that Smad3, a key Smad protein for the TGF-ß antiproliferative responses, is a major physiological substrate for cyclin-dependent kinases CDK4 and CDK2. Except for the Rb family members, Smad3 is the only CDK4 substrate demonstrated so far. CDK phosphorylation of Smad3 inhibits its transcriptional activity and antiproliferative function. Since cyclin D1 that activates CDK4 and the related CDK6 is amplified or overexpressed in a high percentage of human breast cancers, we examined whether Smad3 is highly phosphorylated by CDK in breast cancers. Using primary human breast cancer tissue microarrays and a phosphopeptide antibody against a CDK phosphorylation site in Smad3, we have found that overexpression of cyclin D1 in primary human breast cancer is strongly associated with immunohistochemical staining on the CDK phosphorylation site in Smad3. Thus, diminishing Smad3 activity by CDK phosphorylation may be an important mechanism for resistance to the growth-inhibitory effects of TGF-ß in breast cancers.

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Table of Contents

Introduction

TGF-ß potently inhibits cell cycle progression at the G1 phase (1, 2). Smad proteins play an essential role in mediating the TGF-ß growth-inhibitory responses by regulating the expression of several cell cycle-related genes (2). For example, Smad proteins upregulate the expression of CDK inhibitors p15 and p21 (3-10) and downregulate the expression of the c-myc protooncogene (11-18). We have recently discovered that Smad3, a key Smad protein for TGF-ß growth-inhibitory responses, is a major physiological substrate for G1 cyclin-dependent kinases CDK4 and CDK2 (19, see the attached publication in the appendices). Except for the Rb family, Smad3 is the only CDK4 substrate demonstrated so far. We have further shown that CDK phosphorylation of Smad3 inhibits its transcriptional activity and antiproliferative function (19, see the attached publication in the appendices). The TGF-ß growth inhibitory effects are lost in the vast majority of cancers, including breast cancers (20). Since CDK activity is often high in breast cancers (21, 22), diminishing the activities of Smad3 and presumably the homologous Smad2 by G1 CDK phosphorylation may allow breast cancers to be resistant to the antiproliferative effects of TGF-ß. This grant is directed towards addressing this important issue. In the first year of this grant, we have been analyzing whether the expression levels of certain cell cycle regulators, especially cyclin D1, correlates with Smad3 and Smad2 phosphorylation status in breast cancer cells, and whether introduction of CDK phosphorylation mutant Smad3 can restore TGF-ß responsiveness to breast cancer cells.

Body

Task 2:
We have mapped the CDK4 and CDK2 phosphorylation sites in Smad3 both *in vivo* and *in vitro*. We have generated phosphopeptide antibodies to each of the five sites in Smad3: Thr 8, Thr 178, Ser 203, Ser 207, and S212. We have shown that both CDK4 and CDK2 can phosphorylate each of the five sites *in vitro*. We have further shown that CDK4 and CDK2 phosphorylate three of the five sites: Thr 8, Thr 178, and S212 *in vivo* under physiological conditions. These findings have been published in reference 19 (See the attached publication in the Appendices) and presented in several conferences, including the 95th American Association for Cancer Research (AACR) Annual Meeting held in March, 2004 (See the attached AACR Meeting Abstract #2604 in the Appendices).

The Task 2 of the approved statement of work for this grant is to determine whether high levels of cyclin D1 is correlated with hyperphosphorylation of Smad3 and Smad2 in breast cancer tissue microarrays. Cyclin D activates CDK4 and the related CDK6 (23-26); cyclin D1 is often amplified or overexpressed in many cancers including breast cancers and is oncogenic in breast epithelial cells in a mouse model of breast cancer (21, 22, 27-34). We have analyzed cyclin D1 expression and Thr 178 (T178) of Smad3 phosphorylation level of breast cancer tissue microarrays by immunohistochemical staining. The pT178 phosphopeptide antibody of Smad3 also recognizes the analogous position in Smad2. We have found that overexpression of cyclin D1 in primary human breast cancer is strongly associated with pT178 immunohistochemical staining. Specifically, 87% of ductal carcinoma in situ, primary invasive breast carcinoma, and metastatic carcinoma that overexpressed cyclin D1 were positive for pT178 staining. This is an ongoing collaborative work with my colleague Dr. Michael Reiss at the Cancer Institute of New Jersey, as indicated in my original grant proposal. The data above was obtained from breast cancer tissue microarrays containing biopsy samples from 134 breast cancer patients (34% overexpressed cyclin D1). This work has been presented in the 95th AACR Annual Meeting held in March, 2004 (See the attached AACR Meeting Abstract #291 in the Appendices) and the

Twentieth Annual Meeting on Oncogene held in June, 2004 (See the attached Abstract in the Appendices).

In approximately one third of breast cancers of the 134 cases above, pT178 was stained in the absence of overexpressed cyclin D1. Since cyclin E activates CDK2 and is overexpressed in certain breast cancers (21, 22, 35-38), we examined whether cyclin E overexpression accounted for some of these cases. Thus far, all the cyclin E antibodies we tested did not work in the immunohistochemical staining. We will explore various ways to overcome this technical difficulty. In addition, we have shown that T178 is mostly phosphorylated by CDK and to a less extent by MAPK (19, see the attached publication). We will examine whether MAPK phosphorylation also accounts for some of the phosphorylation in these cases.

As described above, the CDK phosphorylation sites in Smad3 include T8, T178, and S212. The immunohistochemical staining by pT8 phosphopeptide antibody has not been done yet, as the recently cut breast cancer tissue microarray slides were not very good. As soon as good slides are available, we will perform immunohistochemical studies using the pT8 phosphopeptide antibody of Smad3. The pS212 phosphopeptide antibody is extremely difficult to make. We have attempted five times with somewhat differently designed phosphopeptide antigen sources. Thus far, none of the pS212 phosphopeptide antibodies is good enough for the immunohistochemical staining. Because the S212 is a very good CDK phosphorylation site (See the attached publication), we are still attempting to make better antibodies for this site. This part of the work has been done under my NIH grant.

Smad2 is highly homologous to Smad3. It has overlapping as well as distinct functions as Smad3. We have found that Smad2 can also be phosphorylated by CDK4 and CDK2 *in vitro* (See the attached publication). We are conducting research to demonstrate that Smad2 is also a physiological substrate for CDK and we are generating phosphopeptide antibodies to the potential CDK phosphorylation sites in Smad2. This part of the work has been done under my NIH grant. Once the Smad2 phosphopeptide antibodies are available, they will be used for this breast cancer research project.

Task 1:
The Task 1 of the approved statement of work for this grant is to determine whether TGF-ß resistance is correlated with high levels of cyclin D1 and hyperphosphorylation of Smad3 and Smad2 in breast cancer cell lines. We have used the following breast cancer cell lines: BT-474 (39) that is derived from human primary breast adenocarcinomas, MCF-7 (40), MDA-MB-231, MDA-MB-435, and MDA-MB-453 (41) that are derived from malignant pleural effusions. We have confirmed that these breast cancer cell lines are resistant to the TGF-ß growth-inhibitory effects, as reported previously by my collaborator Dr. Michael Reiss (42). We have examined cyclin D1 levels in these cell lines. The cyclin D1 is expressed at high levels in MCF-7, MDA-MB-453, MDA-MB-231, and BT474 cells, and is also expressed at relatively high levels in MDA-MB-435 cells. As a control, cyclin D1 is expressed at low levels in normal breast epithelial cells, such as the HBL100 cells, which are isolated from human breast milk. We also examined Smad3 and Smad2 expression levels as well as the CDK phosphorylation status of Smad3 at the T8, T178 and S212 sites in these cells. Significant non-specific background bands were present in a number of experiments, which prevents us from drawing conclusions. We are modifying the experimental conditions, and we fully expect that this technical problem will be solved. We will soon redo those experiments as well as perform other assays under Task 1.

Task 3:

The Task 3 of the approved statement of work for this grant is to determine whether introduction of CDK phosphorylation resistant Smad3 and Smad2 can restore TGF-ß responsiveness to breast cancer cells. As described above, we have mapped the CDK phosphorylation sites to T8, T178 and S212 in Smad3. We have generated a mammalian expression plasmid for Smad3 that simultaneously mutated these three phosphorylation sites, designated as Smad3 (Triple Mut). We have shown that in transient report gene assays this triple mutant, and to a varying extent, each of the single mutant of the CDK phosphorylation sites (T8V, T178V or S212A) has a higher activity than wild type Smad3 to upregulate the CDK inhibitor p15 and to downregulate c-myc at basal level as well as in the presence of TGF-ß when introduced into $Smad3^{-/-}$ mouse embryonic fibroblasts (MEF). Moreover, we have generated retroviral vector encoding wild type Smad3, each of the CDK phosphorylation mutant (T8V, T178V, or S212A), or the triple mutant. These retroviral forms of Smad3 were used to infect $Smad3^{-/-}$ MEF. We have found that the Smad3 (Triple Mut), and to a varying extent, each of the single mutant of the CDK phosphorylation sites (T8V, T178V or S212A) has a higher activity than wild type Smad3 to inhibit cell proliferation, accompanied by increased p15 level and decreased c-myc expression as analyzed by Northern blot and immunoblotting, respectively. All these results were presented in Matsuura et al., 2004 (See the attached publication).

We have analyzed in the transient transfection assays whether introduction of Smad3 (Triple Mut) and each of the single mutant (T8V, T178V, or S212A) is more potent than wild type Smad3 to activate p15 and to downregulate c-myc when transfected into the various breast cancer cell lines, including the BT-474, MCF-7, MDA-MB-231, MDA-MB-435, and MDA-MB-453 cells. We have observed in some experiments that it is indeed the case. However, for reasons that we still don't understand, the reporter gene assays were not very reproducible in these breast cancer cell lines. We are in the process to clarify this issue.

To determine whether the CDK phosphorylation resistant Smad3 (Triple Mut) can restore TGF-ß responsiveness to the breast cancer cells, we intended to analyze whether the Smad3 (Triple Mut) has a higher activity than wild type Smad3 to activate p15 and to down-regulate c-myc at endogenous level in the absence as well as in the presence of TGF-ß, accompanied by decreased cell proliferation. For this purpose, we attempted to infect the various breast cancer cell lines, including the BT-474, MCF-7, MDA-MB-231, MDA-MB-435, and MDA-MB-453 cells, with the retroviral wild type Smad3 or the CDK phosphorylation resistant Smad3 (Triple Mut). In particular, we attempted a number of times to infect the MDA-MB-453 cells, as the Smad3 level is relatively low in this cell line, which facilitates the comparison of Smad3 (Triple Mut) with Smad3 (wild type). Unlike the mouse embryonic fibroblasts, which we can reach greater than 90% infection efficiency using the ecotropic retrovirus, the infection efficiency of these human breast cancer cells using the amphotropic retrovirus is extremely low, indicated by the absence of the green fluorescent protein (GFP) expression, which is encoded on the sample plasmid as Smad3 separated by the internal ribosome entry site (IRES). We will attempt the various ways to overcome this technical difficult, including trying different methods to increase the retrovirus titer and infection efficiency, such as inclusion of the polytropic envelope protein VSV-G to increase the infection efficiency. Alternatively, we need to generate stable breast cancer cell lines that overexpress the wild type or CDK phosphorylation resistance Smad3.

Key Research Accomplishments

- We have mapped the CDK4 and CDK2 phosphorylation sites in Smad3 and have found that cyclin D1 overexpression in human primary breast cancer is strongly associated with the phosphorylation of one of the sites analyzed.

Reportable Outcomes

Abstracts:

#291 of the Proceedings of the 95th AACR Annual Meeting (Orlando, Florida, March, 2004)
Overexpression of cyclin D1 in primary human breast cancer is associated with linker phosphorylation of receptor-regulated Smads
Wen Xie, Fang Liu, and Michael Reiss

#2604 of the Proceedings of the 95th AACR Annual Meeting (Orlando, Florida, March, 2004)
Inhibition of Smad transcriptional activity and antiproliferative function by CDK phosphorylation
Fang Liu, Isao Matsuura, Natalia G. Denissova, Guannan Wang, Jianyin Long, and Dongming He

#634 of the 43rd Society of Toxicology Annual Meeting (Baltimore, Maryland, March, 2004)
Inhibition of Smad transcription activity and antiproliferative function by CDK phosphorylation
Fang Liu

Page 3 of the Proceedings of the Twentieth Annual Meeting on Oncogene (Frederick, Maryland, June, 2004)
Inhibition of Smad antiproliferative function by CDK phosphorylation
Fang Liu, Isao Matsuura, Natalia G. Denissova, Guannan Wang, Jianyin Long, Dongming He, Wen Xie, and Michael Reiss

Presentations:

The 95th AACR Annual Meeting (Orlando, Florida, March, 2004)
Overexpression of cyclin D1 in primary human breast cancer is associated with linker phosphorylation of receptor-regulated Smads
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The 43rd Society of Toxicology Annual Meeting (Baltimore, Maryland, March, 2004)
Inhibition of Smad transcription activity and antiproliferative function by CDK phosphorylation
Fang Liu

National Institute on Aging/NIH (Baltimore, Maryland, March, 2004)
Inhibition of Smad transcription activity and antiproliferative function by CDK phosphorylation
Fang Liu

National Cancer Institute/NIH (Bethesda, Maryland, June, 2004)
Inhibition of Smad3 antiproliferative function by CDK phosphorylation
Fang Liu

The Twentieth Annual Meeting on Oncogene (Frederick, Maryland, June, 2004)
Inhibition of Smad antiproliferative function by CDK phosphorylation
Fang Liu, Isao Matsuura, Natalia G. Denissova, Guannan Wang, Jianyin Long, Dongming He, Wen Xie, and Michael Reiss

Conclusions

TGF-ß potently inhibits cell proliferation and the early steps of tumorigenesis. Smad proteins mediate the TGF-ß antiproliferative responses. More than 90% of human cancers, including breast cancers, have lost the TGF-ß growth-inhibitory responses by mechanisms that are not clear. Genetic inactivation of the TGF-ß receptors or Smad proteins occurs at a relatively low frequency (~10%) and therefore cannot explain the broad loss of TGF-ß sensitivity in cancer cells. We have recently discovered that CDK4 and CDK2 phosphorylate Smad3 and consequently inhibit its antiproliferative function. Cyclin D1, which activates CDK4 and the related CDK6, is amplified or overexpressed in a high percentage (30-50%) of human breast adenocarcinomas. Based on these observations, we hypothesize that inactivation of Smad proteins by CDK phosphorylation allows breast cancer cells to become resistant to the growth-inhibitory effects of TGF-ß. This grant is directed towards addressing this fundamental issue. We have found that cyclin D1 overexpression is indeed strongly associated with the phosphorylation of one of the CDK phosphorylation sites we analyzed, suggesting that diminishing Smad activity by CDK phosphorylation may be an important mechanism for TGF-ß resistance in breast cancers.

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Appendices

with HKL[24] or XDS[25]. Crystals belong to space group $P2_12_12_1$ with unit cell dimensions $a = 56.7$ Å, $b = 67.7$ Å and $c = 135.6$ Å, and contain three molecules per asymmetric unit.

Structure determination

Selenium sites were located with SnB[26]. MAD phases were calculated with SHARP[27] and improved by density modification. A CID model was built with O[28] and refined with CNS[29]. The refined model has excellent stereochemical quality and a free *R*-factor of 25.2%. A difference Fourier for the peptide-soaked crystal was calculated with phases from the CID model and identified strong density for the CTD peptide bound to one CID molecule (chain B). The two other CID molecules pack against each other, burying their peptide-binding sites. After peptide building, the CID–CTD model was refined to a free *R*-factor of 25.7%. Peptide binding does not result in significant conformational changes in the CID domain. Soaking experiments with phosphoserine did not reveal any additional electron density.

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Cyclin-dependent kinases regulate the antiproliferative function of Smads

Isao Matsuura[1,2,3], Natalia G. Denissova[1,2,3]*, Guannan Wang[1,2,3]*, Dongming He[1,2,3], Jianyin Long[1,2,3] & Fang Liu[1,2,3]

[1]*Center for Advanced Biotechnology and Medicine, and* [2]*Susan Lehman Cullman Laboratory for Cancer Research, Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854, USA*
[3]*Cancer Institute of New Jersey, New Brunswick, New Jersey 08903, USA*

* These authors contributed equally to this work

Transforming growth factor-β (TGF-β) potently inhibits cell cycle progression at the G1 phase[1,2]. Smad3 has a key function in mediating the TGF-β growth-inhibitory response. Here we show that Smad3 is a major physiological substrate of the G1 cyclin-dependent kinases CDK4 and CDK2. Except for the retinoblastoma protein family[3,4], Smad3 is the only CDK4 substrate demonstrated so far. We have mapped CDK4 and CDK2 phosphorylation sites to Thr 8, Thr 178 and Ser 212 in Smad3. Mutation of the CDK phosphorylation sites increases Smad3 transcriptional activity, leading to higher expression of the CDK inhibitor p15. Mutation of the CDK phosphorylation sites of Smad3 also increases its ability to downregulate the expression of c-*myc*. Using Smad3$^{-/-}$ mouse embryonic fibroblasts and other epithelial cell lines, we further show that Smad3 inhibits cell cycle progression from G1 to S phase and that mutation of the CDK phosphorylation sites in Smad3 increases this ability. Taken together, these findings indicate that CDK phosphorylation of Smad3 inhibits its transcriptional activity and antiproliferative function. Because cancer cells often contain high levels of CDK activity[5,6], diminishing Smad3 activity by CDK phosphorylation may contribute to tumorigenesis and TGF-β resistance in cancers.

Cell cycle progression from G1 to S phase is governed by CDK4 and the homologous CDK6 as well as CDK2 (ref. 4). CDK4 and CDK6 are activated by D-type cyclins at early to mid-G1 phase, whereas CDK2 is activated by E- and A-type cyclins during late G1 and S phase, respectively[4]. The activities of CDK4 and CDK6, and of CDK2, are constrained by the p16 INK4 family and the p21 Cip/Kip family inhibitors, respectively[4].

TGF-β potently inhibits cell proliferation by causing cell cycle arrest at the G1 phase[1,2]. Smad proteins can mediate TGF-β growth inhibitory responses[1,2]. In the basal state, Smad2 and Smad3 are distributed throughout cells. In response to TGF-β, Smad2 and

Smad3 are phosphorylated at the carboxyl terminus by TGF-β receptor and form complexes with Smad4 (refs 1, 2). These complexes then accumulate in the nucleus and regulate the transcription of target genes that include cell cycle regulators[1,2], such as the CDK inhibitors p15 and p21 and the protooncogene c-*myc* (refs 7–16). Smad3 is important in antiproliferative responses. For instance, hyperproliferation is a component of the carcinogenic process that leads to the development of metastatic colon cancer in Smad3$^{-/-}$ mice[17]. The loss of Smad3 expression increases susceptibility to tumorigenicity in human gastric cancer[18]. Mice lacking Smad3 display squamous hyperplasia in the stomach (C.-X. Deng, personal communication). Smad3$^{-/-}$ mice also show accelerated wound healing, partly owing to an increased rate of re-epithelialization[19]. A variety of primary cells from Smad3$^{-/-}$ mice are resistant to the growth inhibitory effects of TGF-β (refs 20–22), indicating that Smad3 has a key function in responsiveness to TGF-β.

Because Smads contain potential CDK phosphorylation sites, we analysed whether they are substrates for CDKs. To investigate whether cyclin D–CDK4 can phosphorylate Smads, we performed an *in vitro* kinase assay with endogenous CDK4 immunoprecipitated from Mv1Lu mink lung epithelial cells by an affinity-purified CDK4-specific antibody. Full-length Smad proteins fused to glutathione *S*-transferase (GST) were used as substrates. Two frequently used fusion proteins of retinoblastoma protein (Rb) with GST were included as positive controls. As shown in Fig. 1a, the highly homologous Smad2 and Smad3 were phosphorylated. Smad4, which is homologous to Smad2 and Smad3 in the amino-terminal and C-terminal domains but divergent from Smad2 and Smad3 in the middle proline-rich region, was not phosphorylated (Fig. 1a), nor was GST alone (data not shown). The phosphorylation was

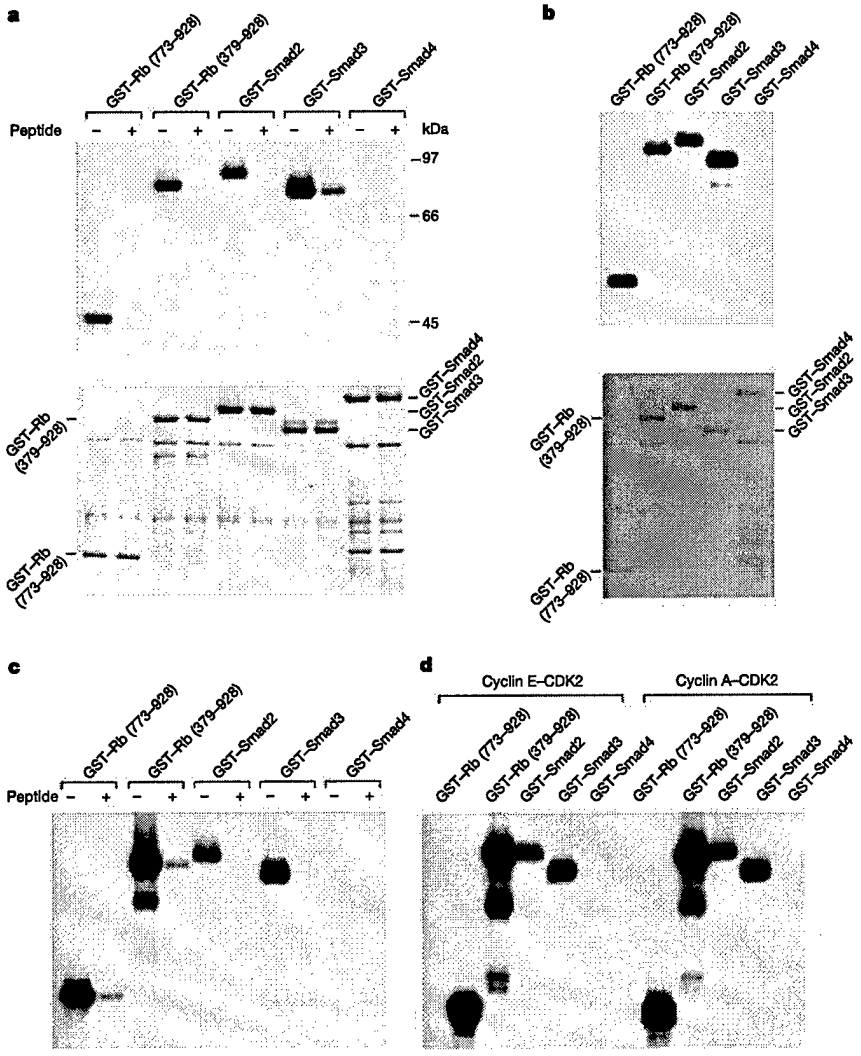

Figure 1 CDK4 and CDK2 can phosphorylate Smad3 and Smad2 *in vitro*. **a**, Top: immunoprecipitated (IP) CDK4 kinase assay. CDK4 immunoprecipitated from 240 μg Mv1Lu cell lysate with 1.2 μg CDK4 antibody in the absence or presence of 9 μg of the antigen peptide (mouse CDK4 amino acids 282–303) was used in a kinase assay with 1 μM substrates. Bottom: protein amounts indicated by Coomassie blue staining. **b**, Top: reconstituted CDK4 kinase assay. Reconstituted cyclin D–CDK4 (500 ng) was used to phosphorylate 0.4 μM substrates. Bottom: protein level indicated by Coomassie blue staining. **c**, Immunoprecipitated CDK2 kinase assay. CDK2 immunoprecipitated from 240 μg Mv1Lu cell lysate with 1.2 μg CDK2 antibody in the absence or presence of 6 μg of the antigen peptide (human CDK2 amino acids 283–298) was used in a kinase assay with 1 μM substrates. **d**, Reconstituted CDK2 kinase assay. Reconstituted cyclin E–CDK2 (13 ng) and reconstituted cyclin A–CDK2 (96 ng) were used to phosphorylate 1 μM substrates.

specific, because preincubation of the antibody with the CDK4 antigen peptide before immunoprecipitation significantly inhibited phosphorylation (Fig. 1a). Because CDK4 has a very strict substrate specificity, indicated by its inability to phosphorylate the canonical CDK substrate histone H1 (ref. 4), we further verified that CDK4 can indeed phosphorylate Smads by using bacterially expressed and *in vitro* reconstituted CDK4 (Fig. 1b). Notably, Smad3 was phosphorylated to a greater extent than Rb by immunoprecipitated CDK4 from Mv1Lu cells (Fig. 1a) and many other cell lines (data not shown) as well as by reconstituted CDK4 (Fig. 1b). Further substrate titration experiments comparing Smad3 and Rb phosphorylation by using either immunoprecipitated CDK4 or reconstituted CDK4 confirmed that Smad3 is an excellent substrate for CDK4 (Supplementary Fig. S1 and Supplementary Table S1). Similar experiments indicated that Smad3 and Smad2, but not Smad4, can also be specifically phosphorylated by immunoprecipitated CDK2 (Fig. 1c) and bacterially expressed and *in vitro* reconstituted cyclin E–CDK2 or cyclin A–CDK2 complexes (Fig. 1d).

To determine whether endogenous Smad3 is phosphorylated by G1 CDKs, we synchronized Mv1Lu cells at the G0/G1 phase by contact inhibition as described previously[23]. Cells were then released from growth arrest by plating into fresh medium and, at different time points, were labelled with ^{32}P-orthophosphate and immunoprecipitated by a Smad3-specific antibody to analyse the endogenous Smad3 phosphorylation status. Unlabelled cells prepared in parallel were analysed by flow cytometry to determine the cell cycle distribution at each time point. As shown in Fig. 2a, Smad3 phosphorylation oscillated in a cell-cycle-dependent manner. The maximal phosphorylation of Smad3 occurred at the G1/S

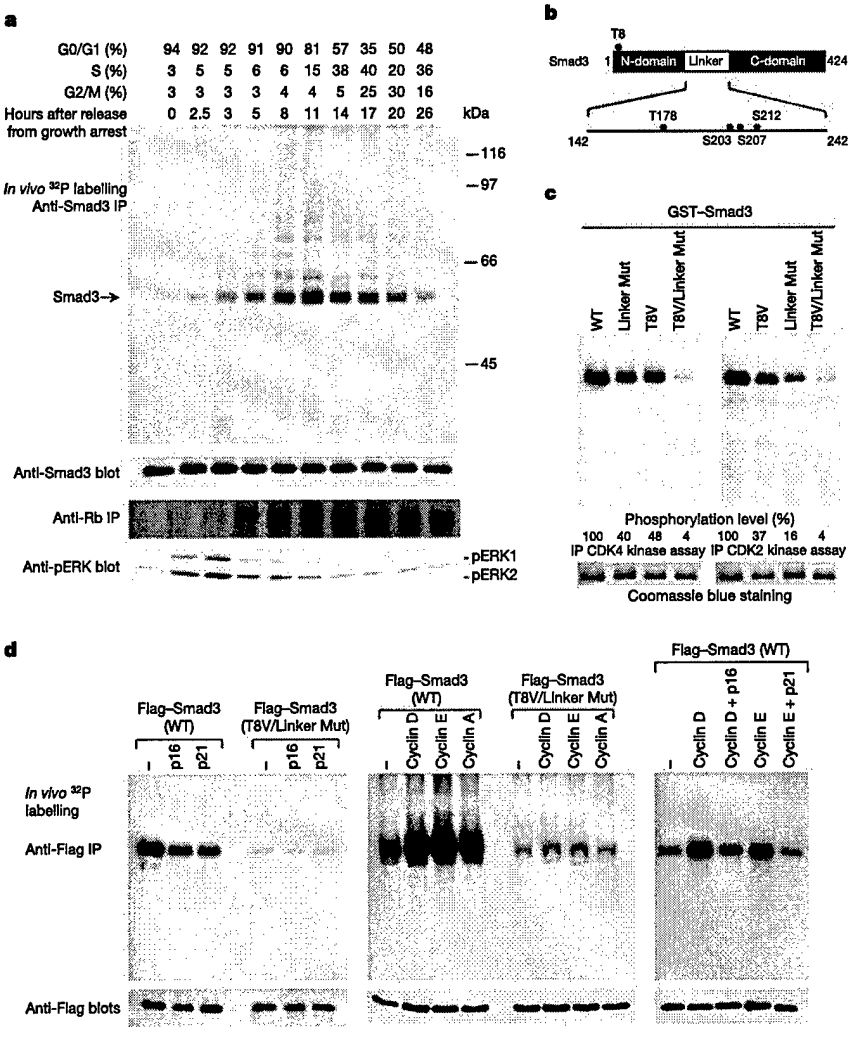

Figure 2 Smad3 is phosphorylated by endogenous G1 CDKs *in vivo*. **a**, Endogenous Smad3 is phosphorylated in a cell cycle-dependent manner. Mv1Lu cells were synchronized by contact inhibition, then plated into fresh medium, labelled with ^{32}P-orthophosphate, immunoprecipitated (IP) first with a Smad3 antibody and subsequently with an anti-Rb antibody. The ERK activity profile, analysed by a phosphotyrosine antibody that recognizes only activated ERK (pERK1 and pERK2) in unlabelled lysates, indicates that ERK might contribute to Smad3 phosphorylation at very early, but not at the peak, phosphorylation time points. **b**, Schematic diagram of Smad3 structure. **c**, Mutation of the Thr 8 and four phosphorylation sites in the Smad3 linker region markedly decreases CDK4 and CDK2 phosphorylation *in vitro*. WT, wild-type. **d**, Left, p16 or p21 transfected into Mv1Lu/L17 cells can inhibit endogenous CDK to phosphorylate Smad3. Middle and right, cyclins transfected into COS cells can activate endogenous CDKs to phosphorylate Smad3, and p16 and p21 can inhibit this activity.

junction, indicating that Smad3 was phosphorylated by G1 CDKs. Subsequent immunoprecipitation of the ^{32}P-labelled cell lysates with an antibody against Rb showed that the peak of Rb phosphorylation slightly lagged behind that of Smad3 phosphorylation (Fig. 2a), suggesting that Smad3 is a good substrate for CDK4 *in vivo*.

Smad3 contains nine potential CDK phosphorylation sites, four of which are located in the proline-rich linker region: Thr 178, Ser 203, Ser 207 and Ser 212 (Fig. 2b and Supplementary Fig. S2). The threonine at amino acid position 8 is potentially a good site, particularly for CDK4 (ref. 24). Through a number of mutational studies, we found that simultaneous mutation of Thr 8 and the four sites in the linker, designated Smad3 (T8V/Linker Mut), markedly decreased phosphorylation by CDK4 or CDK2 (Fig. 2c), indicating that CDK4 and CDK2 phosphorylation might occur within these five sites *in vitro*.

In transient transfection assays, Flag–Smad3 was phosphorylated, and p16 and p21 each inhibited the phosphorylation (Fig. 2d, left panel). Smad3 (T8V/Linker Mut) was phosphorylated to a much lower level than the wild-type Smad3. To determine whether the introduction of exogenous cyclins can activate endogenous CDK to phosphorylate Smad3, the wild-type or mutant version of Flag–Smad3 was cotransfected either individually or together with cyclins D, E or A. As shown in Fig. 2d (middle panel), the phosphorylation of wild-type Flag–Smad3 was significantly increased by cotransfected cyclins. The effects of cyclins D or E can be inhibited by cotransfected p16 or p21, respectively (Fig. 2d, right panel). In contrast, phosphorylation of Smad3 (T8V/Linker Mut) was only slightly increased by cyclins D or E. These observations provide additional evidence that Smad3 is phosphorylated by CDK4 and CDK2 *in vivo* and that the phosphorylation occurs within these five sites.

To determine the exact CDK phosphorylation sites in Smad3, we generated phosphopeptide antibodies against each of the five potential phosphorylation sites: Thr 8 and the four sites in the linker (Thr 178, Ser 203, Ser 207 and Ser 212). Each of the five sites was phosphorylated by both CDK4 and CDK2 *in vitro*, and only Thr 8, Thr 178 and Ser 212 were phosphorylated by CDK4 and CDK2 *in vivo* (Supplementary Figures S3–S5). To confirm that the other four potential sites (Thr 131, Ser 316, Ser 391 and Ser 415) cannot be phosphorylated by CDK, we also generated phosphopeptide antibodies against each of these sites and found that these four sites indeed cannot be phosphorylated by CDK4 or CDK2 (data not shown). Mitogen-activated protein (MAP) kinase was shown to phosphorylate Smad3 in the linker region[25]. We found that Ser 203 and Ser 207 were phosphorylated by MAP kinase and that Thr 178 was phosphorylated mostly by CDK and to a lesser extent by MAP kinase (Supplementary Fig. S5).

To analyse the role of CDK phosphorylation of Smad3, we examined the effect of mutation of the Smad3 CDK phosphorylation sites on the p15 reporter gene. As shown in Fig. 3a, each of T8V, T178V and S212A has a higher activity than the wild-type Smad3 to stimulate the p15 promoter, and the triple mutant (T8V/T178V/S212A) has the highest activity. Moreover, the GAL4–Smad3 triple mutant (T8V/T178V/S212A) has a higher activity than the wild-type GAL4–Smad3 on a GAL4–luciferase reporter gene in the absence as well as in the presence of different concentrations of TGF-β (Fig. 3b). We also found that cotransfection of CDK4 RNA-mediated interference (RNAi) or CDK2 RNAi constructs increases the basal and TGF-β-induced p15 reporter gene activity (Fig. 3c). Taken together, these results indicate that CDK phosphorylation of Smad3 can inhibit its transcriptional activity.

Smad3 also plays a critical role in the downregulation of c-*myc* expression by TGF-β (refs 13–16), which is necessary for subsequent p15 and p21 induction[1]. Mutation of Smad3 CDK phosphorylation sites also increased its ability to downregulate c-*myc* (Fig. 3d). Downregulation of c-*myc* might involve an active repression mechanism, a possibility that requires further investigation.

The above observations prompted us to ask whether Smad3 can inhibit cell cycle progression from G1 to S phase, and whether mutation of the CDK phosphorylation sites renders it more effective in executing this function. Previous studies have shown that Smad3 together with Smad2 and Smad4 activates p15 expression, and introduction of Smad3 into Smad3$^{-/-}$ mouse embryonic fibroblasts (MEF) restores the p15 reporter gene induction by TGF-β (ref. 11). Smad3$^{-/-}$ primary MEF proliferated faster than the wild-type littermate MEF in the ^{3}H-thymidine incorporation assay, and the TGF-β growth-inhibitory effects were largely lost in the Smad3$^{-/-}$ primary MEF (Fig. 4a and ref. 21). To determine whether mutation of the CDK phosphorylation sites enables Smad3 to be more effective at inhibiting G1 cell cycle progression, we generated retroviral vectors that express either the wild-type Smad3 or the

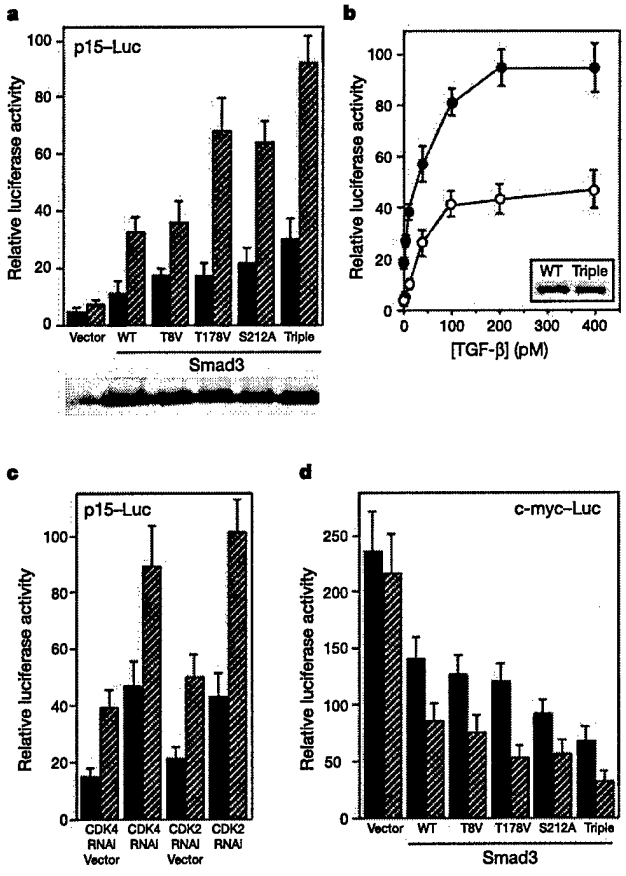

Figure 3 Mutation of CDK phosphorylation sites in Smad3 leads to an increased p15 level and reduced c-*myc* expression in reporter gene assays. **a**, Smad3$^{-/-}$ MEF were transfected with a p15 reporter gene together with the wild-type or various CDK phosphorylation mutants of Smad3. Similar results were obtained in HepG2 cells, and HepG2 cells were used to examine the Smad3 protein expression levels, as shown in the gel at the bottom. Black bars, without TGF-β; hatched bars, with TGF-β. **b**, Mv1Lu/L17 cells were transfected with a GAL4 reporter gene and TGF-β receptor along with either GAL4–Smad3 (open circles; WT) or GAL4–Smad3 (filled circles; Triple Mut), which contains the T8V, T178V and S212A mutations. **c**, CDK4 RNAi or CDK2 RNAi increases p15 reporter gene activity as analysed in HepG2 cells. Black bars, without TGF-β; hatched bars, with TGF-β. **d**, Smad3$^{-/-}$ MEF were transfected with a c-*myc* reporter gene along with the wild-type or various CDK phosphorylation mutants of Smad3 and analysed as indicated. Black bars, without TGF-β; hatched bars, with TGF-β. Error bars show standard deviation (s.d.) of at least three independent transfection results.

various CDK phosphorylation mutants of Smad3, and then used them to infect Smad3$^{-/-}$ primary MEF. As shown in Fig. 4b, wild-type Smad3 increased the cell population in G0/G1 phase and decreased the cell population in S phase. The various CDK phosphorylation mutants of Smad3 augmented these effects. Accordingly, these mutants were more effective than the wild-type Smad3 in inhibiting cell proliferation as measured by the ^{3}H-thymidine incorporation assay (Fig. 4c) and cell number (Fig. 4d), accompanied by increased p15 expression and lower c-Myc levels (Fig. 4e). In addition to the Smad3$^{-/-}$ primary MEF, we have also

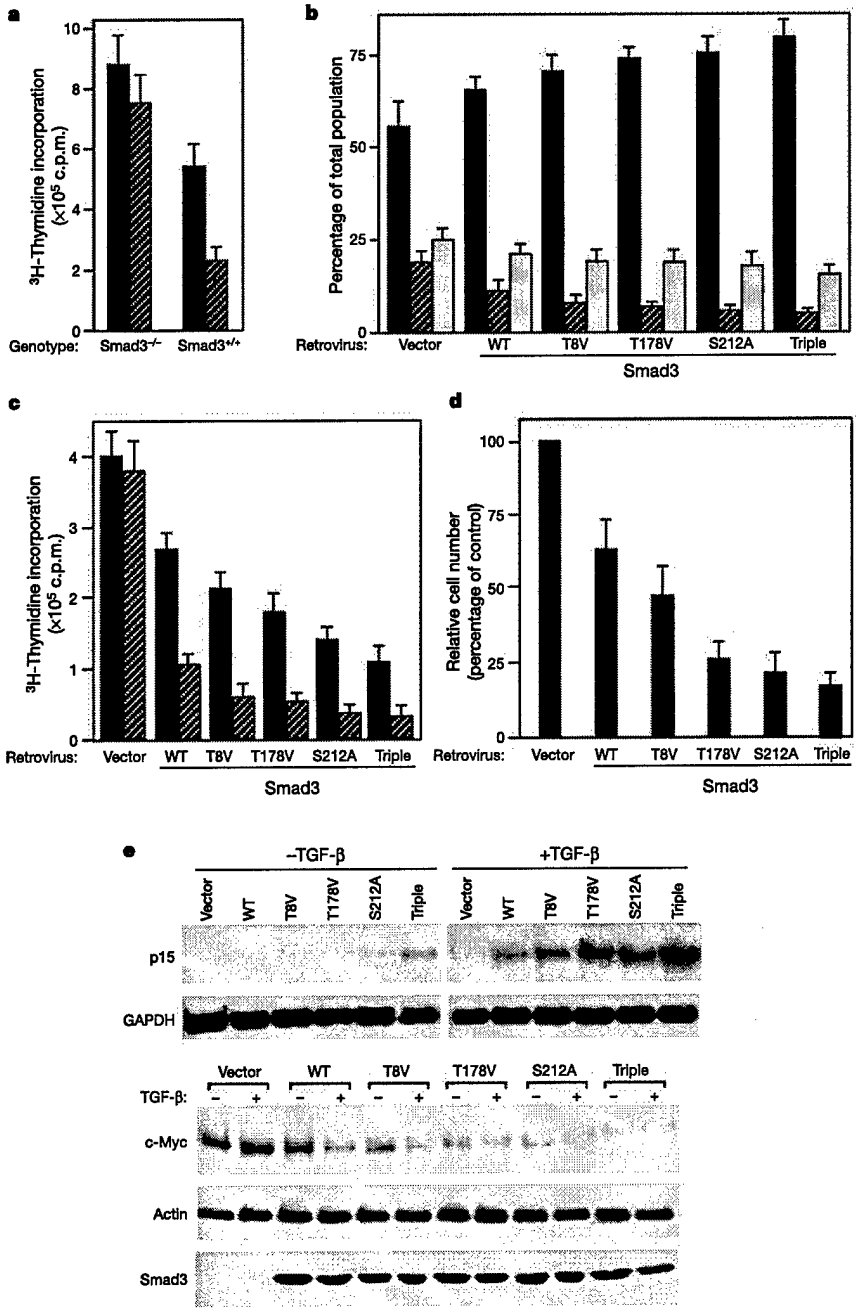

Figure 4 Mutation of the CDK phosphorylation sites in Smad3 leads to increased antiproliferative activities. **a**, Smad3$^{-/-}$ primary MEF and the wild-type littermate control MEF (both at passage 3) were compared in a ^{3}H-thymidine incorporation assay in the absence (black bars) and in the presence (hatched bars) of TGF-β. The average of four experiments is shown. Error bars represent standard deviation (s.d.). **b**–**e**, Smad3$^{-/-}$ primary MEF (passage 3) were infected with retrovirus vector, retroviral wild-type Smad3 or various Smad3 CDK phosphorylation mutants. Infected cells were then split and seeded for fluorescence-activated cell sorting analysis 2 days later (**b**; black bars, G0/G1 phase; hatched bars, S phase; grey bars, G2/M phase), ^{3}H-thymidine incorporation assay (**c**; black bars, without TGF-β; hatched bars, with TGF-β), measurement of cell number about 5 days later (**d**) and analysis of p15 and c-Myc levels (**e**). The error bars in **b**–**d** represent s.d. of four experiments. The infected MEF were treated with TGF-β for 24 h in **e**. Total RNA (10 μg) and protein (15 μg) were analysed by northern blot (top) and immunoblotting (bottom), respectively.

observed the same trend in other cell types including the Mv1Lu epithelial cells, which contain relatively low levels of wild-type Smad3 (data not shown). Taken together, these observations indicate that phosphorylation of Smad3 by CDK facilitates cell cycle progression from G1 to S phase.

Thus, we have shown that Smad3 is phosphorylated by CDK4 and CDK2. Mutation of its CDK phosphorylation sites increases its transcriptional activity and antiproliferative function. We propose that under physiological conditions, phosphorylation of Smad3 by CDK inhibits its transcriptional activity, contributing to a decreased level of p15 and an increased level of c-Myc, thus facilitating cell cycle progression from G1 to S phase. In the presence of physiological concentrations of TGF-β, normal cells are inhibited by TGF-β. However, cancer cells often contain high levels of CDK activities because of frequent amplification, translocation or overexpression of the cyclin D1 gene or inactivation of the tumour suppressor p16 (refs 5, 6). In addition, overexpression of cyclin E and decreases in CDK inhibitor p27 levels also occur in cancer cells[26]. Inactivation of Smad3 and presumably the homologous Smad2 by extensive CDK phosphorylation may provide an important mechanism for resistance to the TGF-β growth-inhibitory effects in cancers. □

Methods

Phosphorylation *in vitro* by immunoprecipitated CDK

Mv1Lu cells were lysed in a buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 0.5% Nonidet P40, 1 mM dithiothreitol (DTT) and protease and phosphatase inhibitors. Antibodies against CDK2 (M2) and CDK4 (C-22) from Santa Cruz Biotechnology were used for immunoprecipitations. The kinase reaction was carried out for 1 h in 30 μl containing 50 mM HEPES pH 7.4, 15 mM $MgCl_2$, 1 mM EGTA, 0.1% Tween 20, 1 mM DTT, 50 μM ATP, 5 μCi [γ-^{32}P]ATP (3000 Ci mmol^{-1}) and substrates at 30 °C. GST–Rb (773–928) contains the proline-rich region, and GST–Rb (379–928) contains in addition the Rb pocket domain.

Phosphorylation *in vitro* by reconstituted CDK

GST–CDK4, GST–CDK2 and His-tagged cyclins D, E and A were expressed and purified from *Escherichia coli* as described previously[27]. GST–CDK and His–cyclin were mixed and incubated with HeLa extracts in the presence of ATP and Mg^{2+} to activate CDK4 or CDK2. Cyclin D–CDK4 was reconstituted as described previously[28] except that no $MnCl_2$ was included. The cyclin D–CDK4 complex was then purified with glutathione agarose beads. Cyclin E–CDK2 and cyclin A–CDK2 were reconstituted as described previously[27] and purified by successive Ni-nitrilotriacetate–agarose and glutathione–agarose chromatography. The purified cyclin–CDK complexes were used to phosphorylate substrates for 40 min in 20 μl reactions containing 35 mM HEPES pH 7.4, 10 mM $MgCl_2$, 1 mM EGTA, 0.1% Tween 20, 1 mM DTT, 15 μM ATP and 5 μCi [γ-^{32}P]ATP for CDK4 or 100 μM ATP and 2 μCi [γ-^{32}P]ATP for CDK2 at 30 °C.

Phosphorylation *in vivo*

For analysis of endogenous Smad3 phosphorylation, Mv1Lu mink lung epithelial cells were synchronized at G0/G1 phase by contact inhibition in complete medium as previously described[23]. In brief, Mv1Lu cells were grown to full confluence in complete medium. Cells were then split and plated into fresh medium. At different time points, cells were phosphate-starved for 0.5 h and then labelled for 1.5 h with 1 mCi ml^{-1} ^{32}P-orthophosphate. Cells were lysed in a buffer containing 10 mM Tris pH 7.8, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P40, and protease and phosphatase inhibitors, and immunoprecipitated by a Smad3-specific antibody. For analysis of transfected Flag-tagged Smad3 phosphorylation, Mv1Lu/L17 or COS cells were transfected by DEAE-dextran. At 30–36 h after transfection, cells were phosphate-starved for 45 min and labelled with ^{32}P-orthophosphate at 1 mCi ml^{-1} for 2.5 h followed by immunoprecipitation by a Flag antibody.

Retrovirus production

Wild-type or CDK phosphorylation mutant Smad3 were subcloned into the retroviral vector pLZRSΔ-IRES–GFP[29] and transfected into ecotropic phoenix packaging cells to produce retroviruses as described previously[30]. Smad3$^{-/-}$ MEF were infected at greater than 90% efficiency.

Generation of MEF and ^{3}H-thymidine incorporation assay

Smad3$^{+/-}$ mice were crossed and MEF were generated as described previously[21]; 2×10^5 Smad3$^{-/-}$ primary MEF and the wild-type littermate control MEF (both at passage 3) were seeded in six-well plates for 24 h, then treated for 24 h with or without 500 pM TGF-β. During the last 4 h, 5 μCi of ^{3}H-thymidine was added to the culture, and ^{3}H-thymidine incorporation was assayed as described previously[21].

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Correspondence and requests for materials should be addressed to F.L. (fangliu@cabm.rutgers.edu).

Supplementary Information

To determine the exact CDK phosphorylation sites in Smad3, we generated phosphopeptide antibodies against each of the five potential phosphorylation sites: T8 and the four sites in the linker (T178, S203, S207 and S212). Each of the phosphopeptide antibodies was raised in rabbits, affinity purified against the phosphopeptide antigen, and cross-absorbed against the unphosphorylated peptide of the same sequence.

We first analyzed these phosphopeptide antibodies in the *in vitro* kinase assay using immunoprecipitated CDK4 and CDK2 from the endogenous Mv1Lu cells and GST-Smad3 as a substrate. The kinase reaction was performed with unlabeled ATP and then subjected to immunoblot with each of the phosphopeptide antibodies. As shown in the Supplementary Fig. S3a, the T8, T178 and S212 sites are phosphorylated by immunoprecipitated CDK4 and CDK2 but not by control IgG immunoprecipitates. When a site is mutated, neither CDK4 nor CDK2 can phosphorylate that site (Supplementary Fig. S3b). Both S203 and S207 can also be phosphorylated by CDK2 and CDK4 in the *in vitro* kinase assay (Supplementary Fig. S3b), however, these two sites are phosphorylated by MAP kinase *in vivo* (see below).

Each of the five phosphopeptide antibodies has a very good specificity as indicated by several other criteria. First, each of the phosphopeptide antibodies recognizes only the wild type Smad3 but not the corresponding mutant Smad3 in Western blot analysis of the transfected Mv1Lu/L17 cell lysates (Supplementary Fig. S4a). Second, each of the phosphopeptide antibodies can recognize overexpressed wild type Smad3 but not the corresponding mutant form in an immunoprecipitation assay (Supplemenatry Fig. S4b). Third, treatment of the phosphorylated

Smad3 with the λ phosphatase leads to the disappearance of the phosphorylated band (Supplementary Fig. S4c). Fourth, the band recognized by each of the phosphopeptide antibodies was Smad3, as none of these antibodies can detect a band that comigrates with Smad3 using cell extracts from Smad3$^{-/-}$ MEF (Supplementary Fig. S4d).

To determine whether the T8, T178, S212, S203 and S207 sites are phosphorylated by CDK4 and CDK2 in a cell cycle-dependent manner, we used HaCaT keratinocytes because they contain relatively high levels of Smad3, which greatly facilitate the detection process. HaCaT cells were synchronized by contact inhibition and serum starvation, and then released into fresh medium. Cells were collected at different time points to analyze the phosphorylation status at each of the sites by immunoblot with the corresponding phosphopeptide antibody. The CDK4 and CDK2 kinase activities were also analyzed in parallel. Since MAP kinase was shown to phosphorylate Smad3 within the linker region[1], MAP kinase activity was also analyzed. As shown in Supplementary Fig. S5a, the T8 and S212 phosphorylation profiles are consistent with CDK4 and CDK2 collaborating to phosphorylate these sites. The T178 phosphorylation profile is consistent with it being phosphorylated by MAP kinase first and then phosphorylated to a much greater extent by CDK4 and CDK2. The S203 and S207 phosphorylation profiles are consistent with these two sites being phosphorylated by MAP kinase. These results are supported by the observation that treatment of cells directly with EGF leads to increases in phosphorylation of T178, S203 and S207, but has no effect on T8 and S212 phosphorylation (Supplementary Fig. S5b). Taken together, these results indicate that the T8 and S212 are CDK phosphorylation sites, the T178 is phosphorylated mostly by CDK and to a lesser extent by MAP kinase, and the S203 and S207 are phosphorylated by MAP kinase.

To provide further evidence that the T8, T178 and S212 sites are phosphorylated by CDK4, we examined their phosphorylation status in $CDK4^{neo/neo}$ MEF, which produce very little CDK4 protein, as the CDK4 promoter is replaced with the neo cassette[2]. As shown in Supplementary Fig. S5c, T8, T178 and S212 phosphorylation levels were reduced in $CDK4^{neo/neo}$ MEF compared to the wild type MEF. Conversely, the T8, T178 and S212 phosphorylation levels were increased in MEF cells that carried $CDK4^{R24C/R24C}$. The R24C mutant does not bind p16, resulting in a higher activity than the wild type CDK4 (refs 3, 4). As a control, the S203 or S207 phosphorylation was little affected by the CDK4 status, either $CDK4^{neo/neo}$ or $CDK4^{R24C/R24C}$ (Supplementary Fig. S5c and data not shown). Thus, the T8, T178 and S212 sites are indeed phosphorylated by CDK4.

To confirm that the T8, T178 and S212 sites can be phosphorylated by both CDK4 and CDK2, we used a plasmid-based RNAi construct to target CDK4 or CDK2. T8, T178 or S212 phosphorylation, but not the control S203 or S207 phosphorylation, was significantly reduced in the presence of cotransfected CDK4 RNAi or CDK2 RNAi plasmids (Supplementary Fig. S5d and data not shown). These results further support the notion that T8, T178 and S212 sites are phosphorylated by both CDK4 and CDK2.

We have also analyzed the phosphorylation status of T8, T178 and S212 in response to TGF-ß, which downregulates CDK4 and CDK2 activity. As predicted, TGF-ß treatment significantly reduces the phosphorylation levels of T8, T178 and S212 (Supplementary Fig. S5e).

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Supplementary Figure Legends

Supplementary Figure S1. Smad3 is an excellent substrate for CDK4 *in vitro*.

a, Substrate titration experiment of GST-Smad3 and GST-Rb by immunoprecipitated CDK4.

CDK4 immunoprecipitated from 240 μg Mv1Lu cell lysates with 1.2 μg CDK4 antibody was used in the kinase assay with different concentrations of substrates as indicated.

b, Phosphorylated GST-Smad3 and GST-Rb bands in (a) were excised and counted for radioactivity. The phosphate incorporated was plotted against substrate concentration.

c, Substrate titration experiment of GST-Smad3 and GST-Rb by 460 ng reconstituted cyclin D-CDK4 with different concentrations of substrates as indicated.

Supplementary Figure S2. Smad3 contains potential CDK phosphorylation sites.

The dots on the Smad3 amino acid sequence indicate all the nine putative CDK phosphorylation sites. The shaded area indicates the proline-rich linker region. The four sites in the linker region plus T8 (labeled with large dots) are marked.

Supplementary Figure S3. The T8, T178, S212, S203 and S207 in Smad3 are phosphorylated by CDK4 and CDK2 *in vitro.*

CDK4 and CDK2 were immunoprecipitated from Mv1Lu cells. Rabbit IgG was used for a control immunoprecipitation in (**a**). The immunoprecipitated kinases were subjected to an *in vitro* kinase assay using wild type or a mutant GST-Smad3 as a substrate in the presence of unlabeled ATP. The reaction mixture was then analyzed by immunoblots with the each of the five phosphopeptide antibodies.

Supplementary Figure S4. The pT8, pT178, pS212, pS203 and pS207 phosphopeptide antibodies have very good specificities towards phosphorylated versus unphosphorylated Smad3.

a, Each of the five phosphopeptide antibodies recognizes wild type Smad3 but not the corresponding mutant Smad3 by immunoblot of transfected Mv1Lu/L17 cells. Myc-tagged wild type Smad3 or a mutant Smad3 was used.

b, Each of the five phosphopeptide antibodies can recognize overexpressed wild type Smad3 but not the corresponding mutant Smad3 in an immunoprecipitation assay as analyzed in Mv1Lu/L17 cells.

c, Phosphatase treatment leads to the disappearance of phosphorylated Smad3 at each of the five sites. Myc-tagged Smad3 was transfected into the Mv1Lu/L17 cells and immunoprecipitated by the Myc antibody. The immunoprecipitates were treated with control buffer or with λ phosphatase

(Calbiochem). The immunoprecipitates were then analyzed by immunoblot with the phosphopeptide antibodies.

d, Each of the five phosphopeptide antibodies specifically recognizes Smad3. Cell lysates from $Smad3^{+/+}$ MEF and $Smad3^{-/-}$ MEF of littermates were analyzed by immunoblots with the five phosphopeptide antibodies.

Supplementary Figure S5. The T8, T178, and S212 in Smad3 are phosphorylated by CDK4 and CDK2 *in vivo.*

a, Analysis of T8, T178, S212, S203 and S207 phosphorylation profiles. HaCaT cells were synchronized by contact inhibition and serum starvation. Cells were released into complete medium and harvested at different time points. Nuclear extracts were then prepared and analyzed by immunoblots with the five phosphopeptide antibodies.

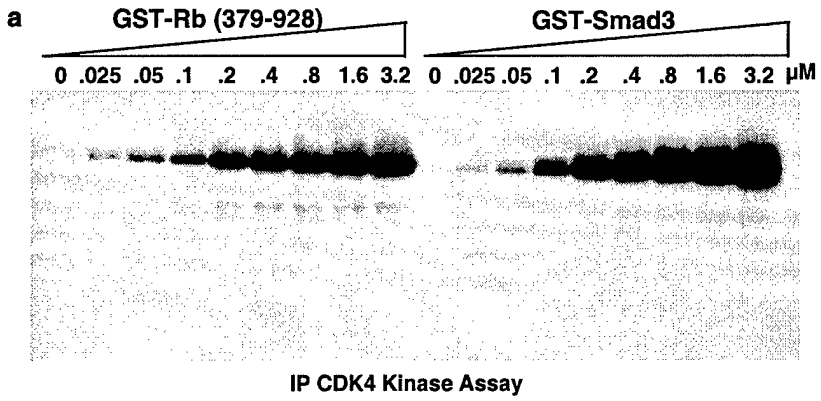
b, EGF treatment increases T178, S203 and S207 phosphorylation but has no effect on T8 and S212 phosphorylation. HaCaT cells were serum-starved for 24 hours, and then treated with EGF (50 ng/ml) for 30 min.

c, Phosphorylation at the T8, T178, and S212 sites of Smad3 is reduced in $CDK4^{neo/neo}$ MEF and is increased in $CDK4^{R24C/R24C}$ MEF.

d, RNAi of CDK4 or CDK2 inhibits T8, T178, or S212 phosphorylation in Smad3. U2OS cells were cotransfected by Myc-tagged Smad3 along with CDK4 RNAi plasmid, CDK2 RNAi plasmid, or the corresponding vector control. The CDK4 RNAi plasmid was constructed in the pSHAG-1 vector[5]. The CDK2 RNAi plasmid was described previously[6].

e, TGF-ß treatment decreases T8, T178 and S212 phosphorylation. Mv1Lu cells were treated with TGF-ß for 24 hours to inhibit the CDK4 and CDK2 activity. The phosphorylation status of T8, T178 and S212 were then determined by immunoblots with the phosphopeptide antibodies.

Supplementary Fig. S1 and Table S1



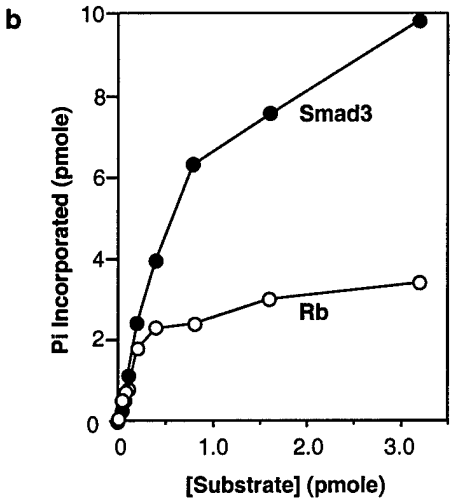
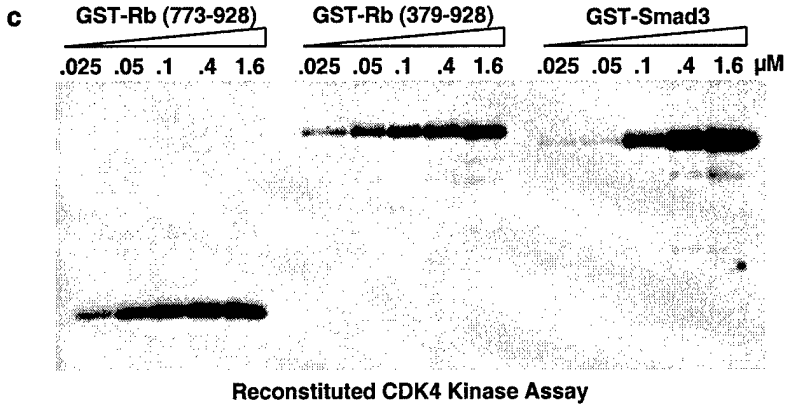


Table S1 Kinetic Parameters of pRB and Smad3 for immunoprecipitated CDK4

Substrate	Km[a]	Vmax[b]
	M	pmole/min
GST-pRB (377-928)	0.3	0.06
GST-Smad3	1.3	0.27

[a] Apparent value

[b] Velocity obtained from immunoprecipitated CDK4 from 240 g Mv1Lu cell lysate



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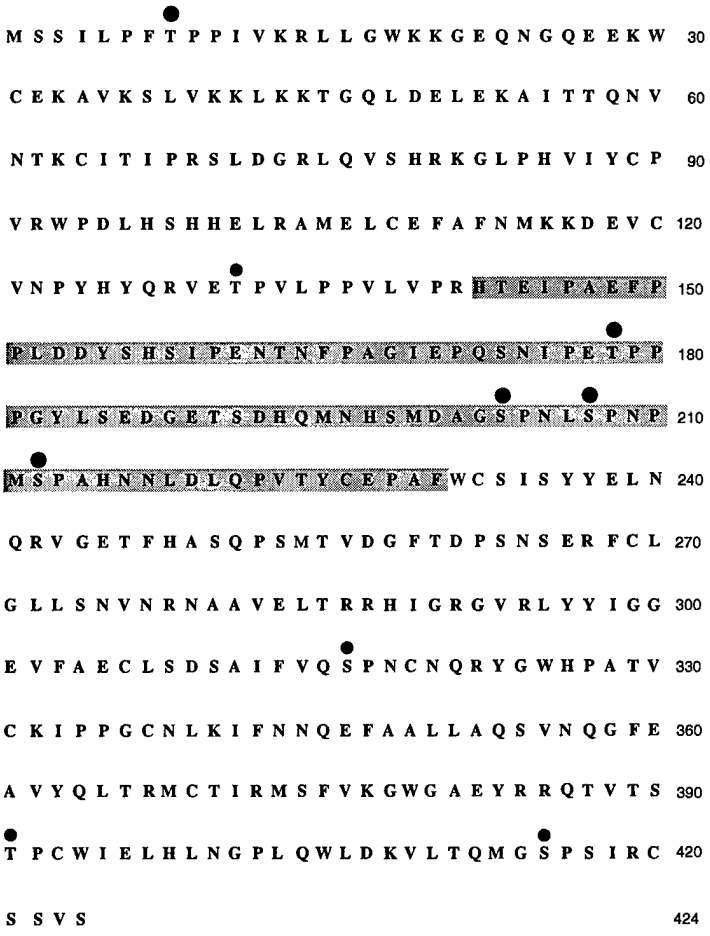

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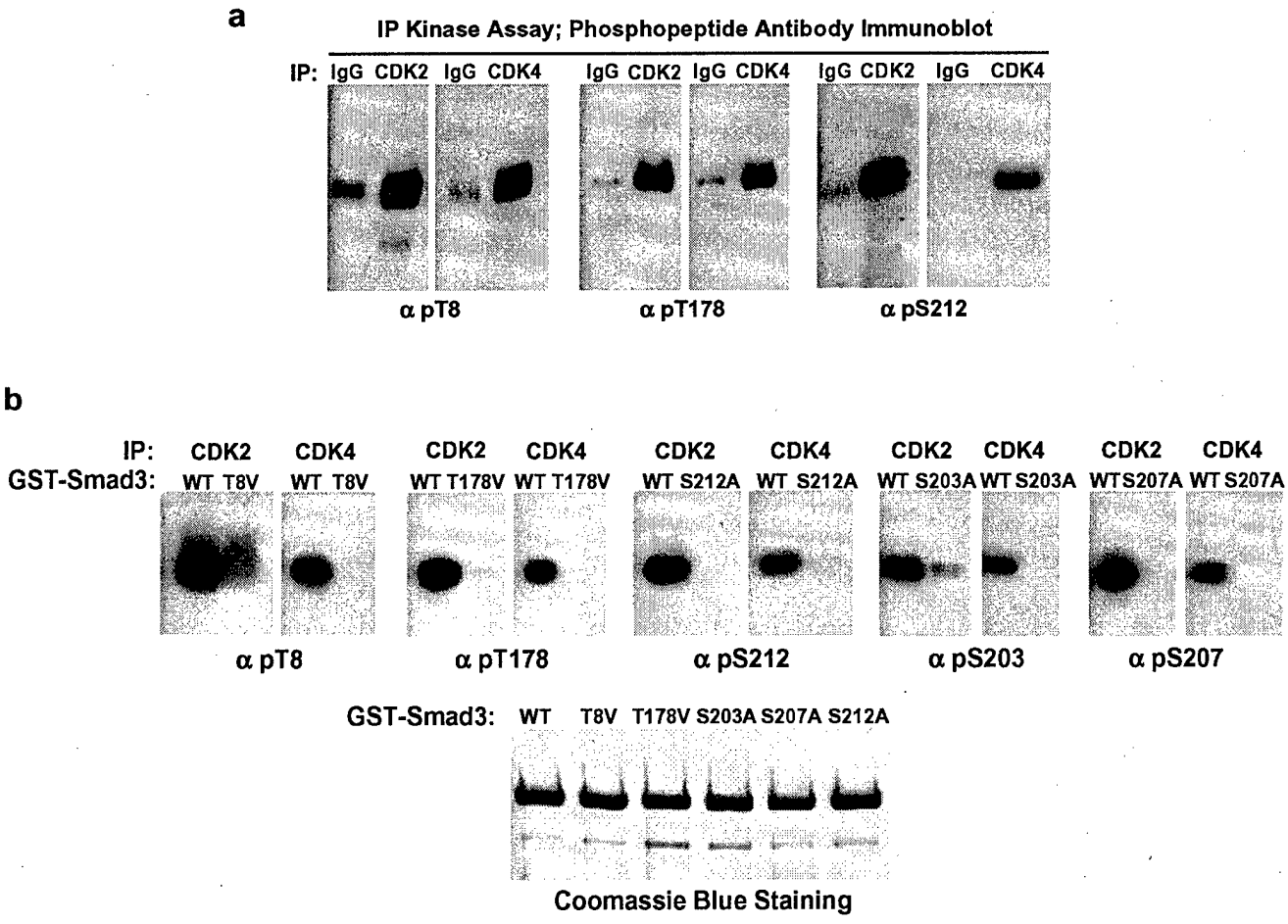
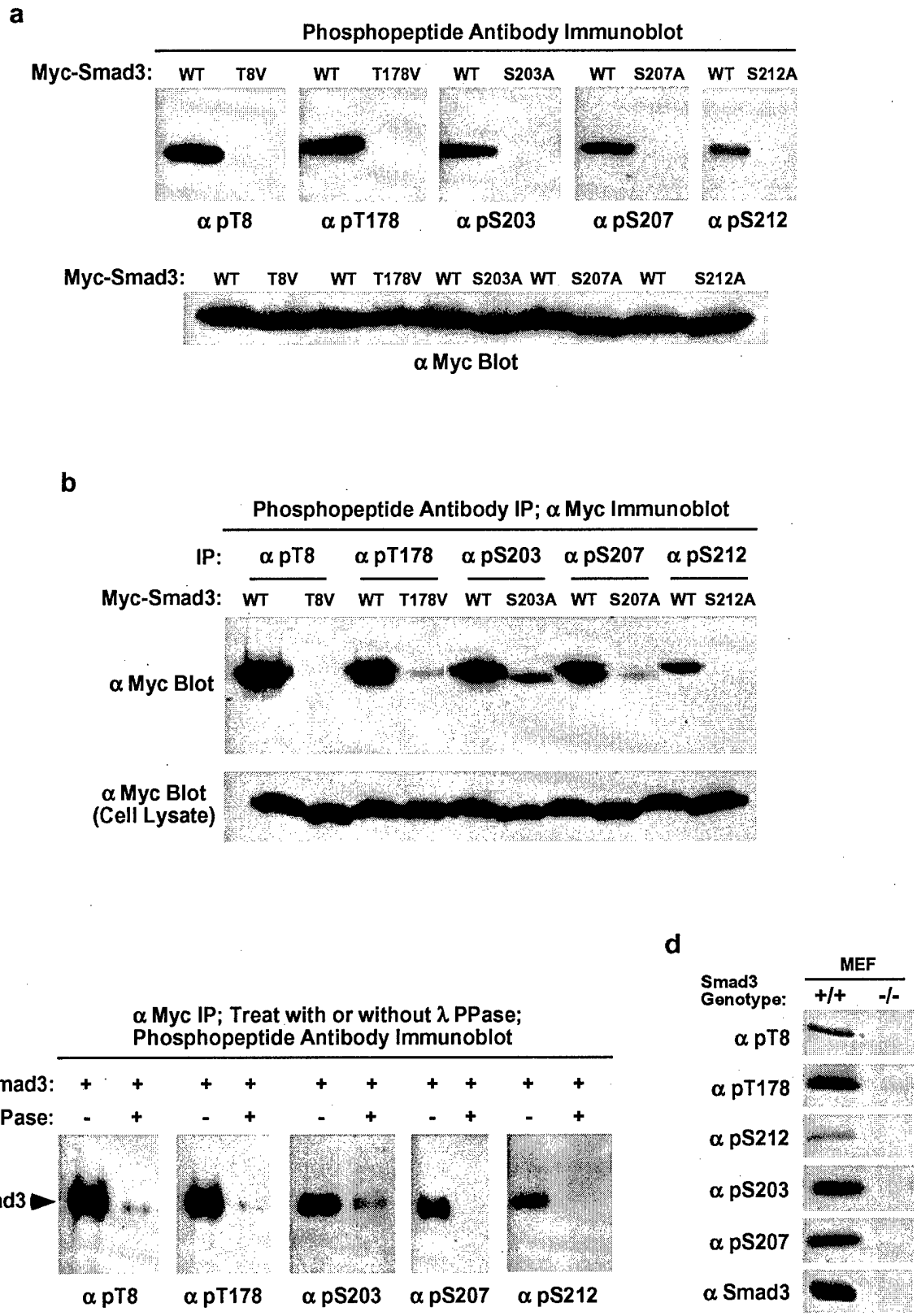
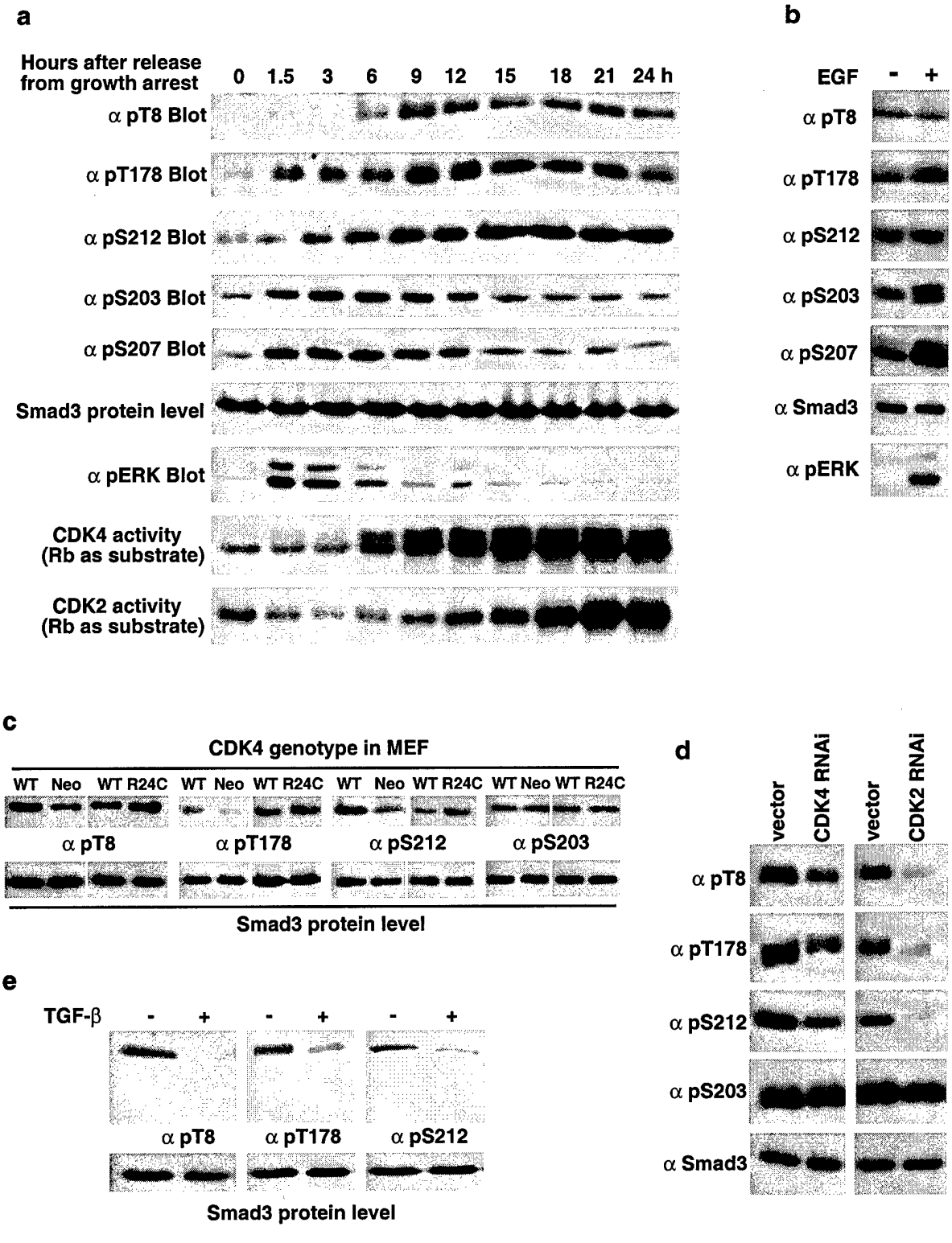


Supplementary Fig. S4



Supplementary Fig. S5



accumulating in the G2 phase of cell cycle. Western blot analysis and kinase assays demonstrated that resveratrol perturbed progression through G2 phase was accompanied by inactivating of p34(CDC2) protein kinase, and an increased level of the tyrosine phosphorylated (inactive) form of p34(CDC2). Furthermore, kinase assay revealed that resveratrol reduced the activity of p34(CDC2) was mediated through the inhibition of CDK7 kinase activity, while CDC25A phosphatase activity was not affected. These results demonstrated that resveratrol induced cell cycle arrest at the G2 phase through inhibiting CDK7 kinase activity.

#2604 Inhibition of Smad transcriptional activity and antiproliferative function by CDK phosphorylation. Fang Liu, Isao Matsuura, Natalia G. Denissova, Guannan Wang, Jianyin Long, Dongming He. *Rutgers University, Piscataway, NJ.*

We demonstrate that Smad3, a key mediator of TGF-beta antiproliferative responses, is a major physiological substrate of G1 cyclin-dependent kinases CDK4 and CDK2. Except for the retinoblastoma protein (Rb) family, Smad3 is the only substrate demonstrated so far for CDK4, and Smad3 can be phosphorylated by CDK4 to a greater extent than Rb. We have mapped both the in vivo and the in vitro CDK4 and CDK2 phosphorylation sites in Smad3. These sites are phosphorylated by CDK4 and CDK2 in vitro and in a cell-cycle dependent manner in vivo. Moreover, we show that their phosphorylation is reduced in CDK4 knockout mouse embryonic fibroblasts (MEF), and their phosphorylation is increased in CDK4 R24C/R24C MEF, which have elevated CDK4 activity. In addition, RNAi experiments also indicate that CDK4 and CDK2 phosphorylate these sites. CDK phosphorylation of Smad3 inhibits its basal as well as TGF-beta induced transcriptional activity, thus preventing activation of the expression of CDK inhibitors p15 and p21. In addition, CDK phosphorylation of Smad3 decreases its ability to downregulate the expression of the protooncogene c-myc. Using Smad3-/- primary mouse embryonic fibroblasts and other epithelial cell lines, we further show that Smad3 inhibits cell cycle progression from the G1 to S phase and that CDK phosphorylation of Smad3 facilitates cell proliferation. Since cancer cells often contain high levels of CDK activities, inactivation of Smad3, and presumably the homologous Smad2 as well, by CDK phosphorylation likely contributes to tumorigenesis and TGF-β resistance in cancers.

#2605 Androgen receptor regulation of cell cycle regulatory proteins in prostate cancer cells. Uma Bai, Eugenia Cifuentes, Evelyn R. Barrack, Prem-Veer Reddy. *Henry Ford Health System, Detroit, MI.*

Our studies revealed that the progression of synchronized LNCaP cells from G1 into S phase is inhibited by the anti-androgen, Casodex (Cifuentes et al., J Cell Physiol 195: 337-345, 2003). In an attempt to understand the role of androgen receptor in transition of prostate cancer cells from G1 into S phase, we looked for changes in expression of cell cycle regulatory genes in synchronized LNCaP cells that were prevented from entering into S phase by Casodex treatment. We employed a cell cycle-specific microarray from Clontech (Catalogue # PT3140-2B) to compare the level of expression of 111 cell cycle-specific genes in Casodex-treated versus control S phase cells. We observed a significant decrease in expression of five genes in cells that were prevented from entering into S phase by Casodex treatment. These genes included Cdc6 (a component of pre-replication complex, pre-RC), RFC37 (37 kDa subunit of DNA replication factor C), RPA70 (70 kDa subunit of DNA replication factor A), cyclin A and cyclin G1. RT-PCR analysis of mRNA isolated from synchronized LNCaP cells revealed that the expression of Cdc6, cyclin G1 and cyclin A is 2 to 3 fold higher in S phase than in G1 phase. Western blot analysis also revealed a significantly higher level of Cdc6, cyclin G1 and cyclin A in S phase cells than in G1 phase. Furthermore, we observed that cyclin G1, which attenuates p53 activity, is overexpressed in prostate tumors compared to nonmalignant prostate. These observations suggest that androgen receptor may regulate the expression of Cdc6, cyclin G1, cyclin A, RFC37 and RPA70 that are essential for the entry of LNCaP cells into S phase. This is the first report of cell cycle dependent regulation of cyclin G1 expression in synchronized prostate cancer cells. Our data implicate cyclin G1 regulated events in S and G2/M phases of prostate cancer cells. (This work is supported by NIH Grant DK 57864).

#2606 Estrogen receptor β inhibits 17β-estradiol activated ERα: A study of the cell cycle and gene expression using T47D and MCF-7 breast cancer cell lines. Johan Hartman, Jinghong Wan, Zahra Mirzaei, Silke Kietz, James S. Foster, Jay Wimalasena, Anders Ström, Jan-Åke Gustafsson. *Karolinska Institute, Huddinge, Sweden and University of Tennessee, Knoxville, TN.*

Estrogen receptor β has been found to counteract the activity of estrogen receptor α in many systems. In agreement with this we find that induced expression of estrogen receptor β in the breast cancer cell line T47D causes inhibition of proliferation at less than equal level of expression of estrogen receptor β compared to estrogen receptor α mRNA. Reduced levels of both mRNA and protein for the G1 to S phase regulating factor cyclin E was found in cells expressing estrogen receptor β, which could explain the inhibition of proliferation. The latter described factor a critical component of the active cdk2 complex important for cell cycle progression through the G1/S-phase transition. Surprisingly, cyclin D1 mRNA and protein were induced earlier in the G1 phase and also reached a higher level when estrogen receptor β was expressed in the cells. Estrogen receptor β mediated down regulation of the cdc25A phosphatase was found but ruled out as the limiting component important for cell cycle progression in these cells. These findings establish a role for estrogen receptor β in breast cancer and imply that estrogen receptor β specific ligands might reduce the incidence of breast cancer. In order to explore the function of ERβ, stable transformants of ERα-positive breast cancer MCF-7 cells with ERβ inducible expression vector were established, the expression of ERβ was confirmed by western blot and real-time PCR, respectively. By using the gene chip technology we found that when ERβ was expressed in synchronized MCF-7 cells treated with 17β-estradiol for 24 hours, expression of a number of genes normally increased by ERα were down regulated. Some examples are thymidine kinase, thymidylate synthetase, trefoil factor 1, and c-myc. The regulated genes strongly indicates that ERβ have an anti-proliferative effect on these cells which is in agreement with the cell cycle analysis of ERβ expressing T47D cells.

#2607 Inhibition of terminal B cell tumor growth by protein kinase C-delta through G2/M arrest and apoptosis induction. Weiqun Li, Hiroshi Miyazaki, Silvio Gutkind. *OPCB, Bethesda, MD.*

Activation of protein kinase C-delta (PKC-δ) regulates many cellular processes involving cell proliferation, differentiation, apoptosis, cell cycle progression, and malignant transformation as determined by the utilization of cell expression systems. Knockout of PKC-δ in mice was shown to result in hyper B cell proliferation, auto-antibody production, and loss of tolerance, suggesting a unique and pivotal role of PKC-δ in negatively regulating B cells and plasma cell growth. To investigate PKC-δ function in terminal B cell transformation, we overexpressed wild type (PKC-δWT) and kinase dead mutant (K376R) in human multiple myeloma (MM) and mouse plasmacytoma (PCT) lines. While PKC-δ overexpressing cells did not exhibit any changes in morphology and growth rate under the normal culture condition, stimulation of PKC-δWT, but not K376R mutant, with 12-O-tetradecanoylphorbol-13-acetate induced G2/M arrest and apoptosis. The PKC-δ mediated effects were selective, since overexpression and activation of PKC-ε in the same cell lines did not induce these phenotypes. The G2/M arrest induced by PKC-δ activation correlated with sustained high levels of cyclin B1 expression, Cdk1 activity, and PKC-δ tyrosine phosphorylation, but did not involve Nek2 inactivation. Together, our results show cell cycle blockade by PKC-δ overexpression and activation in terminal B cell tumors, which inversely correlates with hyper B cell proliferation in PKC-δ knockout mice. Moreover, we demonstrate significant induction of apoptosis in terminal B cell tumors, which was not altered in B cells of PKC-δ knockout mice. Thus, PKC-δ may become a new target in the treatment of MM because of its effects on cell cycle arrest and, particularly, on apoptosis induction.

#2608 Silencing of the p18^{INK4c} gene by promoter hypermethylation in Hodgkin Reed-Sternberg cells in Hodgkin lymphomas. Abel Sanchez-Aguilera, Julio Delgado, Francisca I. Camacho, Margarita Sanchez-Beato, Lydia Sanchez, Carlos Montalban, Manuel F. Fresno, Carmen Martin, Miguel A. Piris, Juan F. Garcia. *Spanish National Cancer Centre (CNIO), Madrid, Spain, Hospital La Paz, Madrid, Spain, Hospital Ramon y Cajal, Madrid, Spain, Hospital Central de Asturias, Oviedo, Spain, Hospital U.C. San Carlos, Madrid, Spain.*

p18^{INK4c} is a cyclin-dependent kinase inhibitor of the INK4 family that interacts with the Rb-kinase activity of CDK6/CDK4. Disruption of p18^{INK4c} impairs B-cell terminal differentiation and confers increased susceptibility to tumor development; however, alterations of p18^{INK4c} in human tumors have rarely been described. Since the neoplastic Reed-Sternberg (RS) cells in Hodgkin Lymphoma (HL) constitute a paradigm of cell cycle deregulation and defective B-cell differentiation, we hypothesized that inactivation of p18^{INK4c} might contribute to the pathogenesis of this neoplasia. We used a tissue microarray approach to analyze p18^{INK4c} expression in 316 HL tumors and in a group of B-cell non-Hodgkin Lymphomas (NHL) and normal lymphoid tissue samples. Nearly half of the HL cases (45%) showed absence of p18^{INK4c} protein expression by RS cells, in contrast with the regular expression of p18^{INK4c} in normal germinal center cells and in normal center-derived NHL. To investigate the cause of p18^{INK4c} repression in RS cells, the methylation status of the p18^{INK4c} promoter was analyzed by methylation-specific PCR and bisulfite sequencing. Hypermethylation of the p18^{INK4c} promoter was detected in 2/4 HL-derived cell lines, but in none of 7 NHL-derived lines. We also detected p18^{INK4c} hypermethylation, associated with absence of protein expression, in 5/26 HL tumors, and confirmed the presence of methylated p18^{INK4c} alleles in microdissected RS cells. Finally, loss of p18^{INK4c} protein

EGFR mutation (EGFRvIII) and compared it to that of ligand activated EGFR expressed at two different levels in the murine fibroblast cell line NR6. Surprisingly it was found that ligand activated EGFR induced the expression of a large group of genes known to be inducible by interferons, particularly interferon gamma. These included genes encoding: suppressor of cytokine signaling 1 and 3 (SOCS-1 and SOCS-3), interferon gamma regulatory factor 1 (IRF-1), interferon inducible GTPase (IIGP), and interferon regulated gene 47 (IRG-47). This property, which EGFR seem to have in common with PDGFR, suggests that these genes are part of a co-regulated module. Expression of this module was absent in the EGFRvIII expressing cell line and the parental cell line. Furthermore, treatment with the specific EGFR inhibitor AG1478, indicated that the regulations were primary, receptor-mediated events. Results were verified by Northern blot analysis. Interferon gamma induced changes in gene expression is mediated mainly through the STAT transcription factors, wherefore the level of STAT activation was investigated downstream of EGFR and EGFRvIII. STAT1 and STAT3 were activated by ligand activated EGFR, whereas EGFRvIII was unable to activate these STATs. Thus, activation of STAT1 and STAT3 correlate with expression of the interferon module and may be responsible for the induction of this module. The time frame and biological significance of activation of STATs and the interferon gamma gene module downstream of EGFR are currently being investigated. An update will be presented.

#289 Lung cancer cells express estrogen receptor (ER) and coactivators and undergo changes in gene expression in response to ER ligands. Pamela A. Hershberger, A. Cecilia Vasquez, Jill M. Siegfried, Mark D. Nichols. *University of Pittsburgh, Pittsburgh, PA.*

Background: The ER is a ligand-activated transcription factor that promotes the proliferation of breast cancer cells. In the presence of estradiol, the ER dimerizes, associates with estrogen response elements in DNA, recruits p160 and p300 transcription coactivators, and alters gene expression. ER antagonists disrupt ER signaling pathways by stimulating the recruitment of corepressors rather than coactivators to DNA sites or by promoting ER degradation. Analogous to their effects in breast cancer cells, ER agonists and antagonists elicit distinct biological responses in non-small cell lung cancer (NSCLC) cells (Stabile et al., Cancer Research 62, 2141-2150 (2002)). Whereas estradiol promotes proliferation, the pure ER antagonist fulvestrant (Faslodex, ICI 182,780) inhibits NSCLC growth *in vitro* and *in vivo*. These studies were undertaken to determine how ER ligands generate such responses in NSCLC cells. **Methods:** We evaluated the expression of ERα, ERβ, and ER coregulators in a panel of normal human lung fibroblasts (NLFB) and NSCLC cell lines by Western blot. Mini-gene arrays containing 96 genes implicated in estrogen signaling were used to explore the capacity of ER ligands to regulate gene expression in human lung cancer cell lines 201T (adenocarcinoma) and 273T (squamous cell carcinoma). Gene expression profiles were generated 24h after treatment with either estradiol (30 nM), fulvestrant (100 nM) or epidermal growth factor (EGF) (30ng/ml), which served as a positive control for a NSCLC growth stimulus in these assays. **Results:** NSCLC cells express ERβ and the p160 coactivator GRIP, but do not express detectable levels of ERα and the p160 coactivator SRC-1. NLFB also express ERβ and GRIP but not ERα. Using mini-gene arrays, we identified several genes that were differentially regulated by estradiol vs. fulvestrant in both 201T and 273T cells including cyclin D1, Id-2, and E-cadherin. These same genes were not affected by treatment with EGF. **Conclusions:** NSCLC cells express proteins implicated in ER signaling and differentially respond to estrogens/anti-estrogens by altering gene expression. The regulation of E-cadherin, cyclin D1, and Id-2 by estradiol but not EGF suggests that expression of these genes does not simply increase in response to a proliferative stimulus, but more likely indicates these genes are specific targets of estrogen in lung cancer cells. It remains to be determined whether cyclin D1 mediates the proliferative response to estradiol in NSCLC, analogous to its action in breast cancer cells. Finally, our data are consistent with a model in which fulvestrant reduces the proliferation of NSCLC cells via its ability to disrupt ER signaling. Fulvestrant may therefore have therapeutic benefit in NSCLC. This work was supported by the NCI SPORE grant CA90440.

#290 Cyclooxygenase-2 induces EP1- and HER-2/Neu-Dependent Vascular Endothelial Growth Factor-C up-regulation: A novel mechanism of lymphangiogenesis in lung adenocarcinoma. Jen-Liang Su, Min-Liang Kuo, Cheng-Chi Chang. *Institute of Toxicology, Taipei, Taiwan Republic of China.*

Cyclooxygenase (COX)-2, the inducible isoform of prostaglandin H synthase, has been implicated in the progression of human lung adenocarcinoma. However, the mechanism underlying COX-2's effect on tumor progression remains largely unknown. Lymphangiogenesis, the formation of new lymphatic vessels, has recently received considerable attention and become a new frontier of tumor metastasis research. Here, we study the interaction between COX-2 and the lymphangiogenic factor, vascular endothelial growth factor (VEGF)-C, in human lung cancer cells and their implication in patient outcomes. We developed an IPTG-inducible COX-2 gene expression system in human lung adenocarcinoma CL1.0 cells. We found that VEGF-C gene expression, but not VEGF-D, was significantly elevated in cells overexpressing COX-2. COX-2-mediated VEGF-C up-regulation was commonly observed in a broad array of non-small cell lung (NSCL) cancer cell lines. The use of pharmacological inhibitors or activators and genetic inhibition by EP receptor-antisense oligonucleotides revealed that prostaglandin EP1 receptor, but not other prostaglandin receptors, is involved in COX-2-mediated VEGF-C up-regulation. At the mechanistic level, we found that COX-2 expression or PGE2 treatment could activate the HER-2/Neu tyrosine kinase receptor through the EP1 receptor -dependent pathway and that this activation was essential for VEGF-C induction. The transactivation of HER-2/Neu by PGE2 was inhibited by way of blocking the Src kinase signaling using the specific Src family inhibitor, PP1 or transfection with the mutant DN-src plasmid. Src kinase was involved in not only the HER-2/Neu transactivation but also the following VEGF-C up-regulation by PGE2 treatment. In addition, immunohistochemical staining of fifty-nine lung adenocarcinoma specimens showed that COX-2 level was highly correlated with VEGF-C, lymphatic vessels density and other clinicopathological parameters. Taken together, our results provided evidence that COX-2 up-regulated VEGF-C and promotes lymphangiogenesis in human lung adenocarcinoma via the EP1/Src/HER-2/Neu signaling pathway.

CELLULAR, MOLECULAR, AND TUMOR BIOLOGY 10: Nuclear Signaling Pathways II

#291 Overexpression of Cyclin D1 in primary human breast cancer is associated with linker region phosphorylation of receptor-regulated Smads. Wen Xie, Fang Liu, Michael Reiss. *Division of Medical Oncology, The Cancer Institute of New Jersey, Robert Wood Johnson Medical School, New Brunswick, NJ and Center for Advanced Biotechnology & Medicine, Department of Chemical Biology (F.L.), Rutgers, The State University of New Jersey, Piscataway, NJ.*

Transforming Growth Factor-β (TGFβ) suppresses breast cancer (BC) development by inhibiting cell cycle progression by causing cell cycle arrest at the G1 phase. Breast cancers (BCs) are typically refractory to TGFβ-mediated cell cycle control. Genetic inactivation of the TGFβ receptors or the post-receptor Smad signaling intermediates accounts for TGFβ resistance in ~10% of BC. However, for the majority of cases, the mechanism of escape from TGFβ-mediated growth control remains unclear. TGFβ inhibits cell cycle progression by down-regulating the expression of cyclin-dependent kinases (CDKs) and c-myc, and by up-regulating the expression of CDK inhibitors p15, p21 and p27. We have recently found that CDK4 and -2 can inactivate Smad signaling by phosphorylation at distinct sites in the linker region of Smad3 and, presumably, Smad2 as well. Given that CDKs are often constitutively activated in BC because of overexpression of cyclin D1 or cyclin E, this may represent an important mechanism of TGFβ signal inactivation. To test this hypothesis, we used an antibody that specifically recognizes Smad3 and -2 phosphorylated in the linker region (pT178) to examine the expression of p(T178)Smad3/2 as a function of cyclin D1 expression by immunohistochemistry in 36 cases of ductal carcinoma in situ (DCIS), 72 cases of primary invasive breast carcinoma (IC), and 18 cases of metastatic carcinoma (MC) assembled in a tissue microarray. 36% of DCIS, 41% of IC, and 33% of MC over-expressed cyclin D1. p(T178)Smad3/2 was clearly detectable in 78%, 70% and 50% of DCIS, IC and MC, respectively. 36%, 37% and 21% of DCIS, IC, and MC, respectively, expressed both p(T178)Smad3/2 and cyclin D1, while 18%, 26% and 36%, respectively, were negative for both p(T178)Smad3/2 and cyclin D1. In aggregate, 87% of DCIS, IC and MC that overexpressed cyclin D1, were positive for p(T178)Smad3/2. Thus, there was a strong association between expression of cyclin D1 and p(T178)Smad3/2 ($p=0.0033$). Conversely, only 49% of DCIS, IC and MC that expressed p(T178)Smad3/2 also overexpressed cyclin D1. These results indicate that cyclin D1 overexpression in primary human BC is strongly associated with linker region phosphorylation of Smad3/2. However, in approximately one third of BC, p(T178)Smad3/2 was expressed in the absence of cyclin D1 overexpression, suggesting that other mechanisms, such as, for example; cyclin E activation, account for p(T178)Smad3/2 expression in these cases. In summary, our results are consistent with the hypothesis that activation of cyclin D1/CDK4 can inactivate TGFβ signaling by linker region phosphorylation in primary human BC.

Inhibition of Smad antiproliferative function by CDK phosphorylation

Fang Liu, Isao Matsuura, Natalia G. Denissova, Guannan Wang, Jianying Long, Dongming He, Wen Xie, and Michael Reiss
Center for Advanced Biotechnology and Medicine, Susan Lehman Cullman Laboratory for Cancer Research, Rutgers University, Cancer Institute of New Jersey, Piscataway, NJ 08854

We demonstrate that Smad3, a key mediator of TGF-ß antiproliferative responses, is a major physiological substrate of G1 cyclin-dependent kinases CDK4 and CDK2. Except for the retinoblastoma protein (Rb) family, Smad3 is the only substrate demonstrated so far for CDK4, and Smad3 can be phosphorylated by CDK4 to a greater extent than Rb. We have mapped both the *in vivo* and the *in vitro* CDK4 and CDK2 phosphorylation sites in Smad3. Mutation of the CDK phosphorylation sites increases Smad3 transcriptional activity, leading to higher expression of the CDK inhibitors p15. Interestingly, mutation of the CDK phosphorylation sites of Smad3 also increases its ability to downregulate the expression of c-Myc. Using $Smad3^{-/-}$ mouse embryonic fibroblasts (MEF) and other epithelial cell lines, we further show that Smad3 inhibits cell cycle progression from the G1 to S phase and that mutation of the CDK phosphorylation sites in Smad3 increases this ability, accompanied by reduced c-Myc and increased p15 levels. Taken together, these findings strongly suggest that CDK phosphorylation of Smad3 inhibits its transcriptional activity and antiproliferative function. Since cancer cells often contain high levels of CDK activities, we examined whether Smad3 is highly phosphorylated by CDK in cancers. Using primary human breast cancer tissue microarrays and phosphopeptide antibodies against the CDK phosphorylation sites in Smad3, we have found that overexpression of cyclin D1 in primary human breast cancer is strongly associated with immunohistochemical staining on the CDK phosphorylation sites in Smad3. Thus, diminishing Smad3 activity by CDK phosphorylation may contribute to tumorigenesis and TGF-ß resistance in cancers.

CURRICULUM VITAE

Name:	Fang Liu
Date of Birth:	December 15, 1962
Place of Birth:	Nanchang, P.R. China
Gender:	Female
Marital Status:	Married
SS#:	032-66-7095
Phone:	732-235-5372
Fax:	732-235-4850
E-mail:	fangliu@cabm.rutgers.edu
Address:	Center for Advanced Biotechnology and Medicine, Rutgers University 679 Hoes Lane, Piscataway, NJ 08854

EDUCATION

1984	B.S., Biochemistry, Peking University, Beijing, China
1992	Ph.D., Biochemistry, Harvard University, under Dr. Michael R. Green

POSTDOCTORAL TRAINING

11/92-11/93	Postdoctoral Fellow with Dr. Michael R. Green Program in Molecular Medicine, University of Massachusetts Medical Center
12/93-4/98	Postdoctoral Fellow with Dr. Joan Massagué Cell Biology Program, Memorial Sloan-Kettering Cancer Center

FACULTY APPOINTMENTS

5/98-	Resident Faculty Member, Center for Advanced Biotechnology and Medicine Assistant Professor, Susan Lehman Cullman Laboratory for Cancer Research Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers University Member, Cancer Institute of New Jersey Graduate Faculty of Molecular Biology and Biochemistry, Rutgers University Graduate Faculty of Cell and Developmental Biology, Rutgers University Graduate Faculty of Cellular and Molecular Pharmacology, UMDNJ/ Robert Wood Johnson Medical School

HONORS AND AWARDS

1985	Ranked first in the field of genetics in the national K.C.Wong Education Foundation Examination in China
1986-1987	K.C.Wong Education Foundation Fellowship
1994-1997	Jane Coffin Childs Memorial Fund for Medical Research Postdoctoral Fellowship
1999-2000	Cancer Institute of New Jersey New Investigator Award
1999-2001	American Association for Cancer Research-National Foundation for Cancer Research Career Development Award
1999-2001	Pharmaceutical Research and Manufacturers of America Foundation Faculty Development Award in Pharmacology
1999-2002	Burroughs Wellcome Fund New Investigator Award in the Toxicological Sciences
2001-2003	Sidney Kimmel Foundation for Cancer Research Kimmel Scholar Award

PUBLICATIONS

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   Genes Dev. *3*, 2083-2090.

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4. Kim, S-J., Wagner, S., **Liu, F.**, O'Reilly, M. A., Robbins, P. D., and Green, M. R. (1992).
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26. **Liu, F.** (2004). Inhibition of Smad antiproliferative function by CDK phosphorylation To be submitted to Cell Cycle

27. Matsuura, I., Wang, G., He, D., and **Liu, F.** (2004). Identification of the MAP kinase phosphorylation sites in Smad3. In preparation

28. Wen, X., **Liu, F.**, and Reiss, M. (2004). Overexpression of cyclin D1 in primary human breast cancer is associated with linker region phosphorylation of Smad3. In preparation

CURRENT RESEARCH SUPPORT

Principal Investigator: Fang Liu
Source: NCI/NIH
Title of Project: Role of Smad proteins in cell growth regulation
Dates of Project: 12/1/01-11/30/06
Total Amount: $1301,798

Principal Investigator: Fang Liu
Source: US Army Congressionally Directed Medical Research Programs (DOD)
Title of Project: TGF-ß resistance in breast cancer
Dates of Project: 7/1/03-6/30/06
Total Amount: $466,500